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Enzymatic decomposition of elicitors of plant volatiles in *Heliothis virescens* and *Helicoverpa zea*

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Abstract

Feeding by larvae of *Heliothis virescens* induces cotton, corn and tobacco plants to release blends of volatile organic compounds that differ in constituent proportions from blends released when *Helicoverpa zea* larvae feed on the same plant species. The same elicitors (and analogs) of plant biosynthesis and release of volatiles, originally identified in oral secretions of *Spodoptera exigua* larvae, were also found in oral secretions of *H. virescens* and *H. zea*. However, relative amounts of these compounds, particularly *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin), 17-hydroxylinolenic acid, and *N*-linolenoyl-L-glutamine, varied among batches of oral secretions, more so in *H. virescens* than in *H. zea*. This variation was due to cleavage of the amide bond of the fatty acid–amino acid conjugates by an enzyme, or enzymes, originating in the midgut. The enzymatic activity in guts of *H. virescens* was significantly greater than that found in guts of *H. zea*. Furthermore, *H. zea* frass contains *N*-linolenoyl-L-glutamine in more than 0.1% wet weight, while this conjugate comprises only 0.003% wet weight in *H. virescens* frass. These results indicated that physiological differences between these two species affect the proportions of volicitin and its analogs in the caterpillars. Whether this causes different proportions of volatiles to be released by plants damaged by each caterpillar species is yet to be determined.

Keywords: Volicitin; Insect-produced elicitors; *Heliothis virescens*; *Helicoverpa zea*

1. Introduction

Plants damaged by herbivorous arthropods release blends of volatile chemicals that attract natural enemies of the herbivores. Such chemically mediated tritrophic plant–insect interactions have been shown for spider mites in a variety of plants (Dicke and Sabelis, 1988; Dicke et al., 1990) and for caterpillars (Turlings et al., 1990; Steinberg et al., 1993). Release of volatiles can be triggered by applying regurgitant from caterpillars to mechanically damaged sites on corn seedlings (Turlings et al., 1990). Elicitors that induce the same blend of volatiles as those induced by caterpillar feeding on cabbage leaves and corn seedlings have been identified as β-glucosidase in the saliva of *Pieris brassicae* caterpillars (Mattiacci et al., 1995) and *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin) in the oral secretions of *Spodoptera exigua* (Alborn et al., 1997).

Oral secretions of *S. exigua* larvae contain several compounds analogous to volicitin, including *N*-linolenoyl-L-glutamine, 17-hydroxylinolenic acid, and linolenic acid as well as the linoleic acid analogs of these compounds (Paré et al., 1998; Alborn et al., 2000). Of these compounds, only *N*-linolenoyl-L-glutamine has been shown to induce a comparable reaction to volicitin in corn seedlings at natural concentrations. When bioassayed at equal concentrations on sweetcorn (variety Ioana), *N*-linolenoyl-L-glutamine is about 30% as active as volicitin (Alborn, unpublished data). Furthermore, Pohnert et al. (1999) found that seven different species of caterpillars, not restricted to *Spodoptera* spp. or the family Noctuidae, also produce volicitin-related compounds; the relative amounts of the conjugates were

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reported to be species-specific. They also reported that *S. exigua* larvae produce only trace quantities of volicitin along with larger amounts of *N*-linolenoyl-L-glutamine and *N*-linoleoyl-L-glutamine, in contrast to the report of Alborn et al. (1997).

De Moraes et al. (1998) found that feeding damage on cotton, corn, or tobacco plants by the tobacco budworm, *Heliothis virescens*, induces the release of different proportions of volatiles from those induced by corn earworm, *Helicoverpa zea*, damage. The specialist parasitic wasp *Cardiochiles nigriceps* exploits these differences to determine which plant is infested by its host, *H. virescens*, in the field. Different odors from plants might be induced by different elicitors or by the same elicitors produced in different proportions by different herbivores. To show whether carnivorous arthropods respond differently to volatiles emitted by plants infested with different herbivore species, as shown in the study of *C. nigriceps*, behavioral studies have been done in different plant–herbivore–carnivore systems. According to these results, some studies support a specificity of herbivore-induced plant volatiles, while others do not (Dicke, 1999, and references therein).

For a better understanding of the specificity of herbivore-induced plant volatiles, detailed analyses of oral secretions of individual herbivore species are necessary, as well as analyses of induced plant volatiles and behavioral responses of parasitic wasps or predators to these volatiles. In the current study, we identified elicitors and analogs in oral secretions of larvae of *H. virescens* and *H. zea*, each of which induces cotton, corn and tobacco plants to release different volatile blends. We also investigated the enzymatic decomposition of the elicitors which results in variability in the relative amounts of the components of the oral secretions.

2. Materials and methods

2.1. Chemicals

N-(17-hydroxylinolenoyl)-L-glutamine (volicitin), *N*-(17-hydroxylinoleoyl)-L-glutamine and *N*-linolenoyl-L-glutamine were synthesized by procedures described by Alborn et al. (2000). These compounds were used as substrates and authentic standards. *N*-Palmitoleoyl-L-glutamine, used as an internal standard, was also synthesized in the same way.

2.2. Insects

Tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), and corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), were obtained from Dr W.J. Lewis, IBPMRL, ARS, USDA, Tifton, GA, and were reared on an artificial pinto bean diet, following the method of King and Leppla (1984). Third instar larvae were fed on cotton leaves for 48 h prior to collection of oral secretions, and for at least 72 h before dissection to obtain fore-, mid-, and hindguts. All larvae were maintained under a 14:10 LD cycle at 60% RH and 25°C.

2.3. Oral secretions

Oral secretions were collected by gently squeezing fourth instar larvae, causing them to regurgitate, as described in Turlings et al. (1993). Each batch of oral secretions consisted of pooled regurgitant collected for half an hour (approximately 50 larvae/batch). After recording the number of larvae and measuring the volume, each batch was immediately stored at −70°C. Before use, the thawed oral secretions were centrifuged at 16,000 ***g*** for 10 min and the supernatant was filtered through 0.45 μm (Millex-HV) and 0.22 μm (Millex-GX) sterile Millipore filters (Millipore, Bedford, MA) to remove bacteria.

The pH of six separate collections of oral secretions from *H. virescens* and *H. zea* was measured using narrow range pH papers (pH 8.0–9.5, Micro Essential Laboratory, Brooklyn, NY). The pH of oral secretions from both insects was consistently in the range of 8–8.5, and thus, pH 8 was subsequently used for all experiments, unless stated differently.

2.4. HPLC analysis

Filtered supernatant of oral secretions was analyzed by HPLC with UV detection at 200 nm (constaMetric 4100 pump, SpectroMonitor 3200 detector, Spectra System AS 3500 autosampler, Thermo Separation Products, Riviera Beach, FL). A reverse-phase column (YMC-Pack ODS-AMQ, 250×4.6 mm ID, YMC, Kyoto, Japan) was eluted (1 ml/min) with a solvent gradient of 20 to 95% CH_3CN (High Purity Solvent, Burdick and Jackson, Muskegon, MI) containing 0.8% acetic acid (Aldrich, Milwaukee, WI), in water (Milli-Q UV PLUS system, Millipore, Bedford, MA) containing 0.5% acetic acid, over 40 min, and then returned to the initial conditions at 45 min. The column temperature was maintained at 60°C. For quantitative analyses, 5 μl of *N*-palmitoleoyl-L-glutamine solution (1 μg/μl) in CH_3CN/H_2O (8:2, v/v) were added to each sample (50 μl) as an internal standard, and then 10 μl of each sample was injected for HPLC analysis.

2.5. Acid methanolysis and chemical analysis

Compounds purified by HPLC were concentrated to dryness under vacuum and treated with methanol/acetic anhydride ($MeOH/Ac_2O$). The procedures described in Mee et al. (1977) were modified in the following way:

Each purified compound was dissolved in 50 μl of MeOH and 50 μl of Ac_2O, and heated at 100°C overnight. After concentration to dryness with a stream of nitrogen, the samples were dissolved in 50 μl of CH_2Cl_2.

The samples were analyzed by GC/MS (6890 Gas Chromatograph equipped with a 30 m×0.25 mm ID, 0.25 μm film thickness HP-5 capillary column, interfaced to a 5973 Mass Selective Detector, Hewlett Packard, Palo Alto, CA). The column temperature was held at 40°C for 1 min after injection and then programmed at 10°C/min to 180°C. The carrier gas was helium at an average velocity of 30 cm/s. Isobutane was used as the reagent gas for chemical ionization, and the ion source temperature was set to 250°C. Compound identifications were confirmed by comparing chromatographic retention times and mass spectra for authentic standards.

2.6. Decomposition of oral secretion components

To measure changes in the composition of *H. virescens* and *H. zea* oral secretions, sterile filtered samples were used. Each sample was divided into five 50 μl-portions. One portion was immediately heated at 95°C for 30 min. The others were incubated at 24°C for 2, 4, 6 or 8 h. After incubation, all samples were heated at 95°C for 30 min and then analyzed by HPLC, as described earlier.

Volicitin-free *H. virescens* oral secretion was prepared by holding a 500 μl filtered supernatant sample at 4°C for about 24 h, until the naturally occurring volicitin had decomposed to below the detection limit when analyzed by HPLC. To learn the basis of this decomposition, three μl (54.7 U) of proteinase K (Fluka Chemical Co., Milwaukee, WI) was added to one of two 30 μl portions of the secretion, and 3 μl of 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 8) was added to a second portion as a control. These samples were then stored at 40°C overnight. Eleven μl of each sample was then added to 99 μl of a synthetic volicitin solution (40 ng/μl) of 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 8). One portion of the mixture (50 μl) was immediately heated for 30 min at 95°C. Another portion (50 μl) was incubated at 24°C for 1 h, then, heated for 30 min at 95°C. After addition of the internal standard, each sample was analyzed by HPLC to quantify the remaining volicitin.

Volicitin-free *H. virescens* oral secretion (60 μl) was also mixed with a 60 μl portion of *H. zea* oral secretion that contained the naturally occurring volicitin (340 ng/μl). One portion of the mixture (50 μl) was immediately heated for 30 min at 95°C. The other portion (50 μl) was incubated at 24°C for 6 h, then heated for 30 min at 95°C. As a control, the volicitin-free *H. virescens* secretion, heated at 95°C for 30 min, was also treated with the *H. zea* secretion in the same manner. After addition of the internal standard, each sample was analyzed by HPLC to quantify the remaining volicitin.

2.7. Determination of the composition of volicitin-related compounds

Six separate collections of *H. virescens* and *H. zea* oral secretions, that had been stored at −70°C were immediately heated at 95°C for 30 min and centrifuged at 16,000 ***g*** for 10 min. The internal standard was added to a portion of the supernatant which then was analyzed by HPLC. The results were calculated as ng of each component/larva, and are presented as mean±SEM of six replications.

2.8. Enzyme preparations

Early fifth-instar larvae of *H. virescens* or *H. zea* were anesthetized on ice and then dissected in saline under a stereo microscope. The fore-, mid-, and hindguts were removed and stored at −70°C. Each gut region was homogenized with 50 mM phosphate buffer (pH 8, 0°C) and centrifuged at 16,000 ***g*** for 10 min. The resulting supernatants were stored at −70°C until used as enzyme preparations.

Midguts were also separated into two parts, midgut contents and midgut tissues. Midgut contents were defined as the food contents of the midgut surrounded by the thin peritrophic membrane; the midgut tissues were defined as the gut wall. Enzyme preparations for midgut contents and tissues were obtained in the same way as described above.

Protein content was determined using the BCA protein assay method (Pierce, Rockford, IL) and bovine serum albumin as a standard.

2.9. Enzyme assay

Enzyme activity was assayed by incubating synthetic volicitin (5.5 μg) with the enzyme preparation (29–86 μg of protein) in a 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 8, total volume 110 μl) with a final substrate concentration of 120 μM. Before the enzyme preparation was added, the mixture was incubated for 30 min at 24°C. After adding the enzyme preparation, the mixture was divided into two samples of 50 μl each. The first sample was immediately heated for 30 min at 95°C to stop the enzymatic reaction. The second sample was incubated for 60 min at 24°C before it was heated for 30 min at 95°C. After addition of the internal standard, each sample was analyzed by HPLC to quantify the remaining volicitin.

To investigate substrate specificity, *N*-linolenoyl-L-glutamine, *N*-(17-hydroxylinoleoyl)-L-glutamine, and *N*-linolenoyl-D-glutamine were each used as a substrate as described above. The enzymatic activities were normalized with the activity for *N*-linolenoyl-L-glutamine, designated as 100% in each case. The resulting activities are presented as the mean±SEM of three replications.

To determine the effect of pH on enzymatic activity, synthetic volicitin was incubated with the enzyme preparation in the following buffers: [0.2 M AcONa–AcOH buffer (pH 5), 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6–8), or 50 mM Glycine–NaOH buffer (pH 9–11)]. The enzymatic activity was determined following the procedures described above. The reaction mixtures were incubated at 24°C for 60 min, and then heated at 95°C for 30 min. Volicitin showed the same solubility over the entire pH range tested; however, the internal standard was not sufficiently soluble below pH 7. Therefore, 50 µl of acetonitrile was added to each 50 µl sample before adding the internal standard. Twenty µl of each sample was injected for HPLC analyses. The activities are shown as relative activities to the largest specific activity at pH 5–11, designated as 100% in each case.

2.10. Analyses of frass extracts

Fresh frass was collected from both species of fourth instar larvae that had fed on cotton leaves overnight. About 50 mg wet-weight of frass was homogenized in 500 µl of acetonitrile/water (1:1, v/v). After centrifuging at 16,000 ***g*** for 10 min, supernatants were analyzed by HPLC on the reverse-phase column as previously described.

2.11. Stereochemistry of glutamine

To determine the stereochemistry of glutamine in the naturally produced elicitors, volicitin and *N*-linolenoyl glutamine obtained from *H. virescens* and *H. zea*, the synthetic forms of these compounds with D- and L-glutamine were analyzed on a chirobiotic T column (250×4.6 mm ID, Advanced Separation Technologies, Whippany, NJ), eluted with 10% CH_3CN in 10 mM $AcONH_4$–AcOH buffer, pH 4.5, at a flow rate of 1 ml/min. The column temperature was maintained at 35°C.

2.12. Statistical analyses

Data were analyzed by the Mann–Whitney *U* test, using JMP, 3.2.6, (SAS Institute, Cary, NC).

3. Results

3.1. Identification of compounds

HPLC analyses of oral secretions from *H. virescens* and *H. zea* showed the presence of the same 8 compounds (Fig. 1). These compounds are identical to the components previously identified in *S. exigua* oral secretions (Alborn et al., 2000). For identification, each compound eluted from the HPLC column was collected

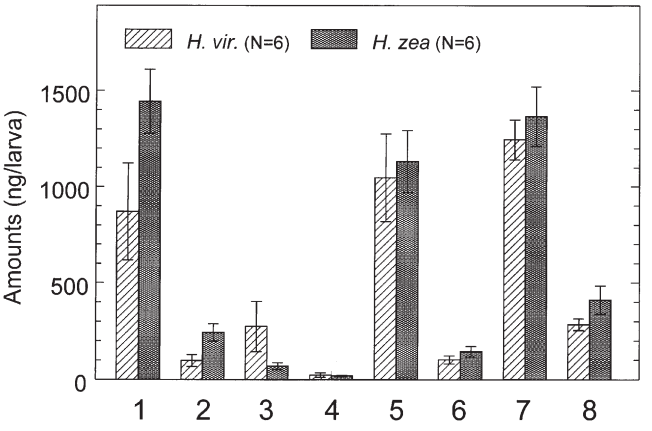

Fig. 1. Mean quantities (±SEM, *n*=6) of compounds found in batches of *H. virescens* and *H. zea* oral secretions analyzed by HPLC-UV at 200 nm, after 48 h larval feeding on cotton leaves. The data was calculated as ng/larva for each batch analyzed (about 50 larvae/batch). Numbers in the figures represent the following compounds: 1, *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin); 2, *N*-(17-hydroxylinoleoyl) glutamine; 3, 17-hydroxylinolenic acid; 4, 17-hydroxylinoleic acid; 5, *N*-linolenoyl-L-glutamine; 6, *N*-linoleoyl glutamine; 7, linolenic acid; 8, linoleic acid.

and treated with MeOH/Ac_2O. The products of this reaction were then analyzed by GC-MS. After this treatment, compound 1 produced three prominent peaks. Chemical ionization (CI) MS analysis of peak 1 (t_R 13.8 min) revealed a prominent (M+1) ion at *m/z* 144, which was identical to methyl pyroglutamate. The CI mass spectrum of peak 2 (t_R 15.3 min) from compound 1 showed an M+1 ion at *m/z* 218, consistent with *N*-acetyl glutamate dimethyl ester. The CI mass spectrum of peak 3 (t_R 25.0 min) from compound 1 showed an (M+1-CH_3COOH) ion at *m/z* 291, which was identical to methyl 17-acetoxylinolenate. Treatment of synthetic volicitin with MeOH/Ac_2O produced the same peaks and mass spectral ions as those shown above. In addition, the t_R of the HPLC analyses indicated that the amino acid moiety was glutamine, not glutamic acid. Thus compound 1 was positively identified as *N*-(17-hydroxylinolenoyl) glutamine.

In the same manner, compounds 2–8 (Fig. 1) were identified as *N*-(17-hydroxylinoleoyl) glutamine, 17-hydroxylinolenic acid, 17-hydroxylinoleic acid, *N*-linolenoyl glutamine, *N*-linoleoyl glutamine, linolenic acid and linoleic acid, respectively. GC retention times and mass spectral data for the products of compounds 1–8 treated with MeOH/Ac_2O are summarized in Table 1.

Analyses of compounds 1 and 5 on the chiral column gave retention times of 3.24 and 6.30 min, respectively, which are identical to the synthetic compounds with the L-glutamine moiety. These results identified compounds 1 and 5 as *N*-(17-hydroxylinolenoyl)-L-glutamine and *N*-linolenoyl-L-glutamine, respectively. The retention times of the synthetic compounds with the D-glutamine moiety were 4.60 and 10.55 min, respectively.

Table 1
GCMS data for compounds 1–8 treated with MeOH/Ac_2O[a]

Compound	GC peak (t_R, min)	Diagnostic ion (*m/z*)	Component
1	13.8	144 (M+1)	Methyl pyroglutamate
	15.3	218 (M+1)	Dimethyl *N*-acetylglutamate
	25.0	291 (M+1-CH_3COOH)	Methyl 17-acetoxylinolenate
2	13.8	144 (M+1)	Methyl pyroglutamate
	15.3	218 (M+1)	Dimethyl *N*-acetylglutamate
	25.8	293 (M+1-CH_3COOH)	Methyl 17-acetoxylinoleate
3	25.0	291 (M+1-CH_3COOH)	Methyl 17-acetoxylinoleate
4	25.8	293 (M+1-CH_3COOH)	Methyl 17-acetoxylinoleate
5	13.8	144 (M+1)	Methyl pyroglutamate
	15.3	218 (M+1)	Dimethyl *N*-acetylglutamate
	21.3	293 (M+1)	Methyl linolenate
6	13.8	144 (M+1)	Methyl pyroglutamate
	15.3	218 (M+1)	Dimethyl *N*-acetylglutamate
	21.2	295 (M+1)	Methyl linoleate
7	21.3	293 (M+1)	Methyl linolenate
8	21.2	295 (M+1)	Methyl linoleate

[a] Numbers represent the following compounds: 1, *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin); 2, *N*-(17-hydroxylinoleoyl) glutamine; 3, 17-hydroxylinolenic acid; 4, 17-hydroxylinoleic acid; 5, *N*-linolenoyl-L-glutamine; 6, *N*-linoleoyl glutamine; 7, linolenic acid; 8, linoleic acid.

3.2. Decomposition of N-(17-hydroxylinolenoyl)-L-glutamine and N-linolenoyl-L-glutamine in H. virescens oral secretions

Analyses of *H. virescens* oral secretions showed more batch-wise variability in compounds 1, 3 and 5 (Fig. 2), compared with the secretions from *H. zea*. For example, sometimes only trace amounts of volicitin (peak 1) were detected in *H. virescens* secretions alongside a relatively large amount of 17-hydroxylinolenic acid (peak 3)(Fig. 2(C)). To determine the reasons for this variability, filtered supernatant of oral secretions of *H. virescens* and *H. zea* were incubated at 24°C for 8 h. Interestingly, changes in composition were observed only in *H. virescens* (Fig. 3). The amount of volicitin in oral secretions of *H. zea* decreased by an average of only 5%, which was not significant, over a period of 8 h at 24°C (n=3). In *H. virescens* samples, the amounts of volicitin and *N*-linolenoyl-L-glutamine decreased, while those of 17-hydroxylinolenic acid and linolenic acid increased quantitatively over the 8 h period. A similar decomposition was also observed for *N*-(17-hydroxylinoleoyl) glutamine and *N*-linoleoyl glutamine.

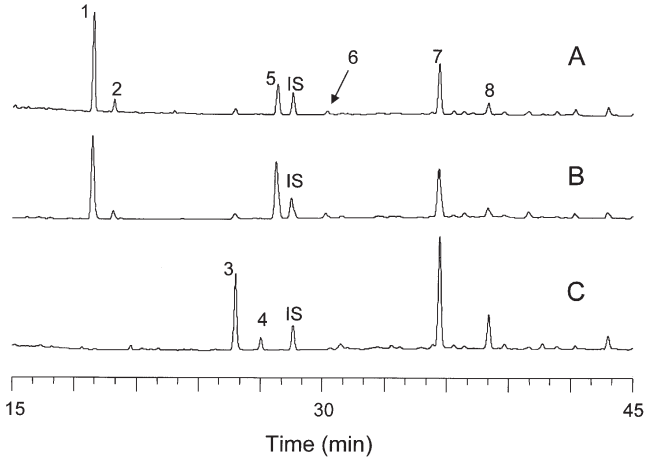

Fig. 2. Typical chromatograms of oral secretions from *H. zea* (A) and from two different *H. virescens* collections (B) and (C). The samples were prepared and analyzed as described in the materials and methods. Chromatograms (HPLC-UV analysis at 200 nm) show the compounds detected in the supernatant of oral secretions from fourth instar *H. zea* and *H. virescens* fed on cotton leaves for 48 h. Numbers represent the same compounds as in Fig. 1. IS, *N*-palmitoleoyl-L-glutamine.

This decomposition was not observed in the secretions boiled at 95°C for 30 min (Fig. 3). Furthermore, 94% of the original amount of volicitin remained in the sample treated with proteinase K, while only 13% was detected in the untreated sample. We concluded that the decomposition was due to the presence of enzymes in *H. virescens* oral secretions.

HPLC analyses of *H. zea* oral secretions treated with "volicitin-free *H. virescens* oral secretions" and incubated at 24°C for 6 h showed only trace amounts of volicitin to be present, while 93% of the original volicitin still remained in the heated control sample. This, coupled with the observation that the composition did not change in untreated oral secretions, suggested that *H. zea* oral secretions lacked the decomposing enzymes.

3.3. Proportions of the compounds in H. virescens and H. zea oral secretions

The mean quantities of the compounds, in ng/larva, for six batches of *H. virescens* and *H. zea* oral secretions are shown in Fig. 1. It was possible to reduce enzymatic

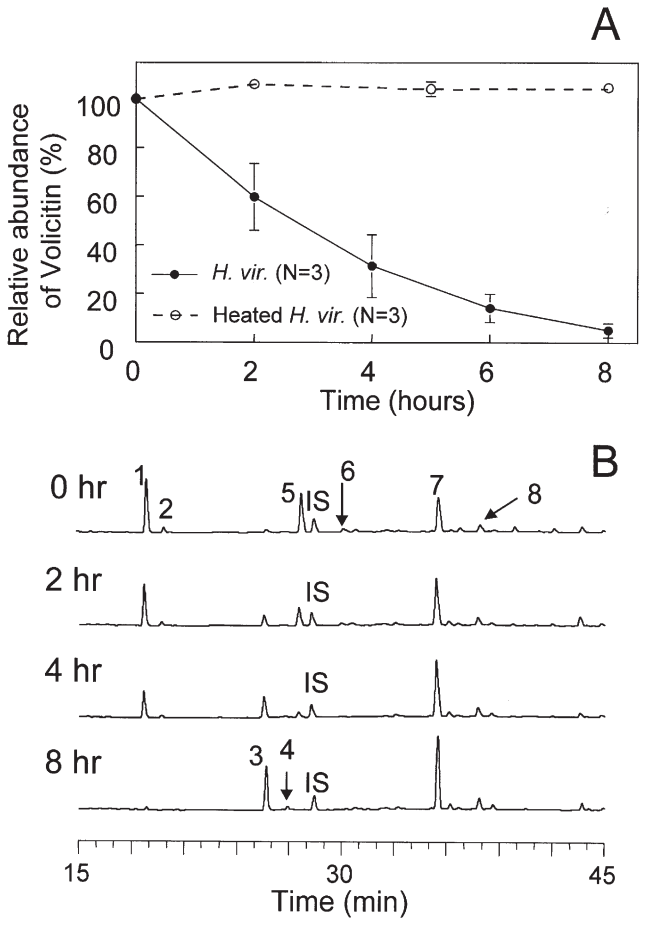

Fig. 3. Decomposition of volicitin in *H. virescens* oral secretions at 24°C over 8 h. (A) Decomposition of volicitin and the effect of heat (95°C for 30 min). The amounts of volicitin were determined by HPLC analyses as described in materials and methods. Each point represents the mean±SEM of three replications and is presented in % of the amount of volicitin at time 0. (B) HPLC chromatograms (UV detection at 200 nm) showing changes in the composition of *H. virescens* oral secretions over time. Numbers represent the same compounds as in Fig. 1. IS, *N*-palmitoleoyl-L-glutamine.

decomposition by heating the oral secretions, although the *H. virescens* oral secretions still showed very large variations among individual samples. Consequently, no significant differences were found in the relative amounts of compounds 1–8 between these two species.

3.4. Enzymatic activities in foregut, midgut and hindgut preparations

Our initial experiments were designed to determine if there were significant differences between *H. virescens* and *H. zea* in the abilities of fore-, mid- and hindgut preparations to decompose volicitin. Although all three preparations from both species decomposed volicitin, the enzymatic activities in the midgut and hindgut of *H. virescens* were significantly greater than those of *H. zea* (Midgut; $P<0.05$, hindgut; $P<0.01$), whereas there was no significant difference in activity in the foregut preparation between the two species (Fig. 4(A)).

Midguts from both insect species were further separated into gut wall and gut contents to determine whether the degrading enzymes were bound to the epithelial membrane or present free in the gut lumen. The midgut tissues showed no significant difference between the two species. The contents from the *H. virescens* showed significantly greater enzymatic activity ($P<0.05$) than those from *H. zea*. This experiment indicated that enzymes that degrade volicitin are present in the midgut lumen, and are apparently not bound to the cells of the gut wall (Fig. 4(B)).

The influence of pH on the enzymatic activity was investigated over the range of pH 5–11. The enzymatic activity at pH 7–8 was higher than at all other pH values in this range (Fig. 5).

3.5. Substrate specificities

Synthetic volicitin, *N*-(17-hydroxylinoleoyl)-L-glutamine, *N*-linolenoyl-L-glutamine and *N*-linolenoyl-D-glu-

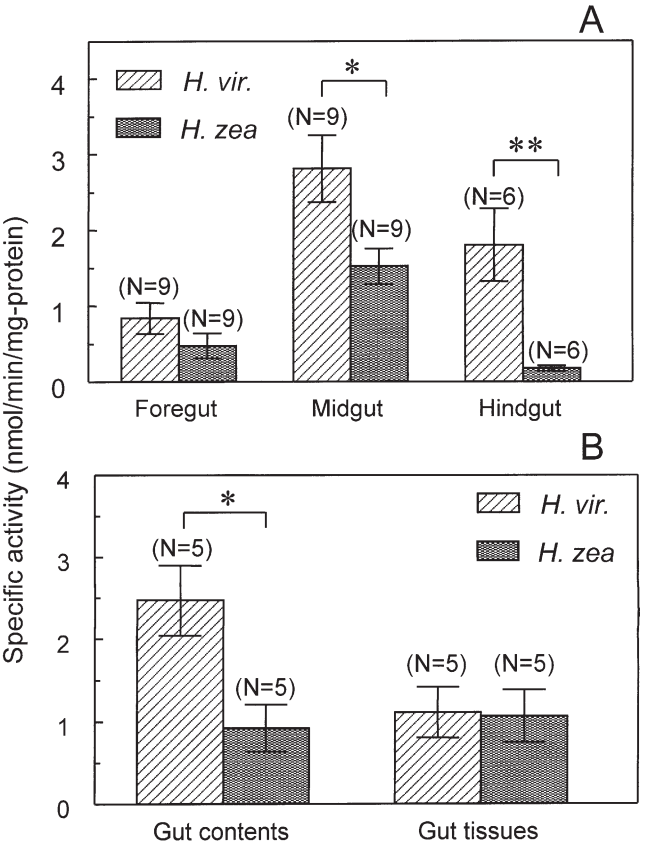

Fig. 4. Decomposition of volicitin by (A) fore-, mid- and hindgut preparations and (B) by midgut contents and midgut tissue preparations from fifth instars of *H. virescens* and *H. zea*. Sample preparation and analyses are described in materials and methods. Each point represents the mean±SEM of 5–9 replications. Asterisks (* $P<0.05$, ** $P<0.01$) indicate statistically significant differences between *H. virescens* and *H. zea* (Mann–Whitney *U* test).

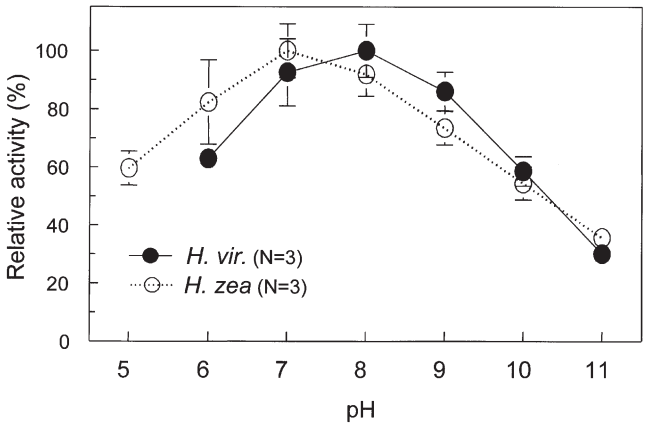

Fig. 5. Influence of pH on the decomposition of volicitin by midgut preparations of fifth instar *H. virescens* and *H. zea*. Each point represents the mean±SEM of three replications.

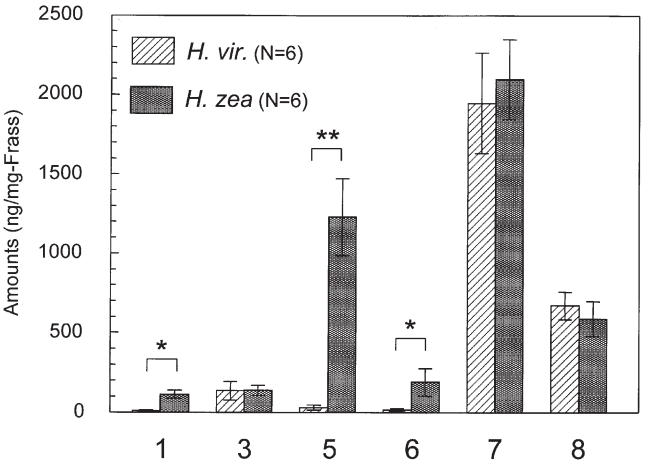

Fig. 6. Composition of extracts of fresh frass from fourth instars of *H. virescens* and *H. zea*. The amounts of the compounds were determined by HPLC-UV analyses at 200 nm. Each point represents the mean±SEM of six replications. Asterisks (* $P<0.05$, ** $P<0.01$) indicate statistically significant differences between *H. virescens* and *H. zea* (Mann–Whitney *U* test). Other differences are not significant. Numbers represent the same compounds as in Fig. 1.

tamine were added to crude midgut enzyme preparations from both species. The relative activities, based on the decomposition for *N*-linolenoyl-L-glutamine (100%), were 94.9±3.9% for volicitin, 95.0±1.7% for *N*-(17-hydroxylinoleoyl)-L-glutamine and 6.0±2.5% for *N*-linolenoyl-D-glutamine in *H. virescens* and 85.8±8.1%, 83.7±3.5% and 7.3±3.3% in *H. zea*, respectively. Taking into account the enantiomeric excess of synthetic *N*-linolenoyl-D-glutamine (81% ee), the enzymes effectively discriminate L-glutamine from D-glutamine. For both insects, the only degradation products that could be detected were the corresponding free fatty acids (17-hydroxylinolenic and 17-hydroxylinoleic acid for volicitin and *N*-(17-hydroxylinoleoyl)-L-glutamine, and linolenic and linoleic acid for *N*-linolenoyl-L-glutamine and *N*-linoleoyl-L-glutamine).

3.6. Volicitin-related compounds found in frass of both species

HPLC analysis of frass extracts revealed the presence of volicitin, 17-hydroxylinolenic acid, *N*-linolenoyl-L-glutamine, *N*-linoleoyl-L-glutamine, linolenic acid and linoleic acid with trace amounts of *N*-(17-hydroxylinoleoyl)-L-glutamine and 17-hydroxylinoleic acid (Fig. 6). In *H. zea*, where the enzymatic activity was observed to be significantly less than *H. virescens*, the frass contained significantly greater amounts of *N*-linolenoyl-L-glutamine ($P<0.01$) and volicitin ($P<0.05$) than the *H. virescens* frass. Interestingly, *N*-linolenoyl-L-glutamine comprised more than 0.1% of wet-weight *H. zea* frass. The amounts of 17-hydroxylinolenic acid, linolenic acid and linoleic acid were, however, not significantly different between the two species.

4. Discussion

Since De Moraes et al. (1998) demonstrated that plants release different blends of volatile chemicals in response to damage by *H. zea* and *H. virescens* larvae and that the parasitoid *Cardiochiles nigriceps* can distinguish between these two blends, the question that arises is how the plants distinguish between the two herbivore species. Although there may be other explanations, including physical or mechanical differences in the way the larvae inflict damage, a logical hypothesis is that the different species produce different elicitors. However, our results show that the oral secretions of both *H. virescens* and *H. zea* contain the same fatty acid–glutamine conjugates found in *S. exigua*. This is consistent with the results of Pohnert et al. (1999) who found that volicitin and the same analogs were present in the oral secretions of larvae of seven different Lepidopterous species.

There are two other possible chemical explanations for the differences in plant response to the two species. The first is differences in chirality of the elicitor molecules. Volicitin has two asymmetric centers, one at the 17th carbon of the linolenic acid chain and the other in the glutamine moiety. We have not yet been able to determine the chirality at the 17th carbon, so there is a possibility that chirality at that position could play a role in determining the response of the plant. However, the synthetic volicitin that is racemic at the 17th carbon appears to be equal in activity to the natural volicitin in eliciting corn seedlings to produce volatiles (Tumlinson et al., 2000). In volicitin and in linolenoyl glutamine only L-glutamine has been found in all the species we have investigated. The second possibility is that different

proportions of volicitin and the analogs elicit different responses by the plants. We have found that *N*-linolenoyl-L-glutamine is about 30% as active as volicitin on corn seedlings (Alborn, unpublished data) and it is possible that some of the other analogs are active on other plants or in certain combinations.

In an attempt to determine why the proportions of the compounds varied to such a great extent in different batches of the oral secretions (Fig. 1), we discovered that the composition of the *H. virescens* oral secretions changed over a period of a few h when allowed to stand at room temperature (Fig. 2). This change involved a decrease and eventual disappearance of the fatty acid–amino acid conjugates with a concomitant increase of the corresponding free fatty acids. Sterile filtering of the oral secretion had no effect on these transformations, indicating that they were not caused by bacteria. On the other hand, heating at 95°C for 30 min (Fig. 2) or treatment with proteinase K stopped the reactions, indicating that they were the result of enzymatic activity.

To determine the source of the degradative enzymes, fore-, mid-, and hindguts of both species were removed, separated, and extracted, and the extracts were incubated with volicitin and other substrates. The midguts of both species had the greatest enzymatic activity and the foreguts the least (Fig. 4(A)). Somewhat surprisingly, given the very low level of degradative enzymatic activity found in *H. zea* oral secretions, *H. zea* midgut specific activity was about half that of *H. virescens*. Further studies indicated most of the midgut activity in *H. virescens* is in the lumen rather than in the gut tissues, whereas there was no significant difference in specific activity between gut tissues and contents of *H. zea* midguts (Fig. 4(B)).

Lepidoptera larvae have a full complement of digestive enzymes, with elevated pH optimally suited to the gut environment. For example, three proteinases have been identified in the midgut fluid of *Bombyx mori* (Eguchi and Kuriyama, 1983). The proteinases are identical to those found in the midgut epithelia, suggesting that they are secreted by the midgut cells. These proteinases have high pH optima of 10.7–11.4. Lenz et al. (1991) showed that proteinases of regurgitated fluids from fifth-instar *H. zea* originate from the midgut lumen, with a possibility of some being contributed by the salivary glands. They reported the pH optima to be 8.0–8.5 for trypsin and 7.5–8.0 for chymotrypsin in crude preparations. In the present study of degradative enzymes obtained from the midgut homogenates of *H. virescens* and *H. zea*, we found pH optima of 7–8 in crude preparations (Fig. 5).

The methods of collecting the secretions that contain the elicitors have raised the question as to where these compounds are produced. Paré et al. (1998) showed that the fatty acid moiety of volicitin originates in the plant on which the caterpillar feeds, while the glutamine comes from the insect. The elicitors may be produced in the oral cavity of the chewing caterpillar, or they may actually be produced at the interface of damaged plant tissue as the fatty acids are liberated from plant membranes by lipases in response to mechanical damage inflicted by the chewing caterpillar. The present study shows that it is very likely that the enzymes in *H. virescens* regurgitant that degrade the fatty acid–amino acid conjugates are due to contamination by midgut content. It is not clear why the *H. zea* oral secretions have less degrading enzyme activity than those of *H. virescens*. When *H. zea* oral secretions were mixed with volicitin-free *H. virescens* oral secretions, the naturally occurring volicitin in the *H. zea* secretions was rapidly decomposed, indicating a lack of inhibition of this type of enzymatic activity in *H. zea*. It is possible that there are structural differences in the guts of the two species which results in different amounts of midgut content being mixed with foregut content when the insects are forced to regurgitate, but the results in Fig. 4(B) also indicate that relatively less enzyme is free in the lumen of *H. zea* midguts. Significantly, the appearance of relatively large amounts of undegraded fatty acid–amino acid conjugate in frass of *H. zea* supports the hypothesis that less of the conjugates are degraded in the *H. zea* guts. The conjugates present in the frass may also play a role in the plants species specific induced response. *H. virescens* and *H. zea* larvae prefer to feed within plant tissue, like stem and fruit, and their frass will be in contact with damaged plant tissue. Preliminary results also showed, not surprisingly, that an extract of 100 μg *H. zea* frass equivalents elicited a strong induced release of volatiles in corn seedlings while an extract of *H. virescens* frass, had no activity. However, it is not clear if, or under which conditions, frass by itself will induce plants to release volatiles.

There are several implications of the results of this study that are important to understanding the interactions between insect herbivores and plants as well as those that involve tritrophic interactions. In the first place, it is difficult to collect oral secretions from caterpillars without introducing artifacts. Furthermore, it is important to deactivate the enzymes in the oral secretions as quickly as possible after collection to avoid changes in composition. Pohnert et al. (1999) reported that *S. exigua* produces only a trace amount of volicitin with large amounts of *N*-linolenoyl-L-glutamine and linoleoyl-L-glutamine, in contrast to previous results (Alborn et al., 1997; Paré et al., 1998). Although this difference might be due to using a different strain of *S. exigua*, it might also be possible that volicitin is decomposed by enzymes in *S. exigua* regurgitant and consequently that differences in methods of collection or handling could result in significant differences in composition of caterpillar oral secretions. We have observed considerable variations in proportions of the components

in different batches of *S. exigua* oral secretions (unpublished). Even after *H. virescens* oral secretions were collected into a vial cooled with dry-ice/acetone to avoid decomposition, the amounts of volicitin in *H. virescens* oral secretions still varied from 76 to 1876 ng/larva, compared to the amounts in *H. zea* that varied from 1009 to 2056 ng/larva. At this point, we do not know what compositions of the volicitin-related compounds interact with damaged plant tissues. We also do not know if differences in proportions of volicitin and analogs have any effect on the volatile blends released by the damaged plants. Although it may be possible to answer the latter question to some extent with synthetic elicitors, it is difficult to duplicate the damage inflicted on a plant by a chewing caterpillar. Since the fatty acid–amino acid conjugates are broken down in the midgut or, as in the *H. zea*, excreted intact in large amounts in the frass, it is not clear if they play any role in digestion in the insects, or if their presence in frass will have an effect on the plant. The answers to these and other questions await further experimentation.

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